Supplemental Material and Methods

MiRNA arrays. Human CD4+ T cells were separated from PBMCs obtained from 6-11 healthy donors and T cell subsets were sorted based on the expression of following surface markers: TH1: CD4+ CD8– CCR5+ CD25– CD45RA–; TH2: CD4+ CD8– CRTh2+ CCR5– CD45RA– CD25–; TH17: CD4+ CD8– CCR6+ CCR4+ CXCR3– CD45RA–; Tregs: CD4+ CD8– CD25hi; naïve: CD4+ CCR7+ CD25– CD45RO–. Total RNA was extracted and as a common reference a pool of total CD4+ T cells from all donors was used. MiRNA arrays were performed and analysed at Exiqon (Denmark). Arrays data are MIAMI compliant and are available at GEO (www.ncbi.nlm.nih.gov/geo) with accession number GSE33946.

Supplemental Figure Legends

Supplementary Figure 1. Mast cells can be efficiently differentiated in the absence of NF-kB p50 and show normal unresponsiveness to repeated LPS stimulation. A) Bone marrow-derived mast cells from control and p50ko mice were differentiated in presence of IL-3 for at least 3 weeks, after which toluidine blue staining of cytospun cells was performed to assess overall morphology and content of cytoplasmic granules. **B)** Control and p50ko mast cells were treated with LPS for 3h (M-L) only or repeatedly for 3h+20h (L-L) or 3h+20h+24h (L-L-L). After stimulation an intracellular cytokine staining was performed to assess expression of the indicated cytokines. Representative of at least four independent experiments. **C)** Same as in (B) except that expression level of miR-146a was assesses by qRT-PCR (M-M=medium, unstimulated; M-L=LPS 3h; L-M=LPS 20h; L-L=LPS 3+20h). **D)** (Top) Western blot for p65 in control and p50ko mast cells. Cells were stimulated with PMA and ionomycin (P+I) for the indicated times, after which nuclei (N) and cytoplasm (C) were separated for protein extraction and Western blot analysis. (Bottom) Nuclear (N) and cytoplasmic (C) extracts were prepared from unstimulated p50ko and control mast cells and p65 expression was assessed by Western blot. β-tubulin was used to quantify expression of p65 in cytoplasmic extracts (numbers below blot).

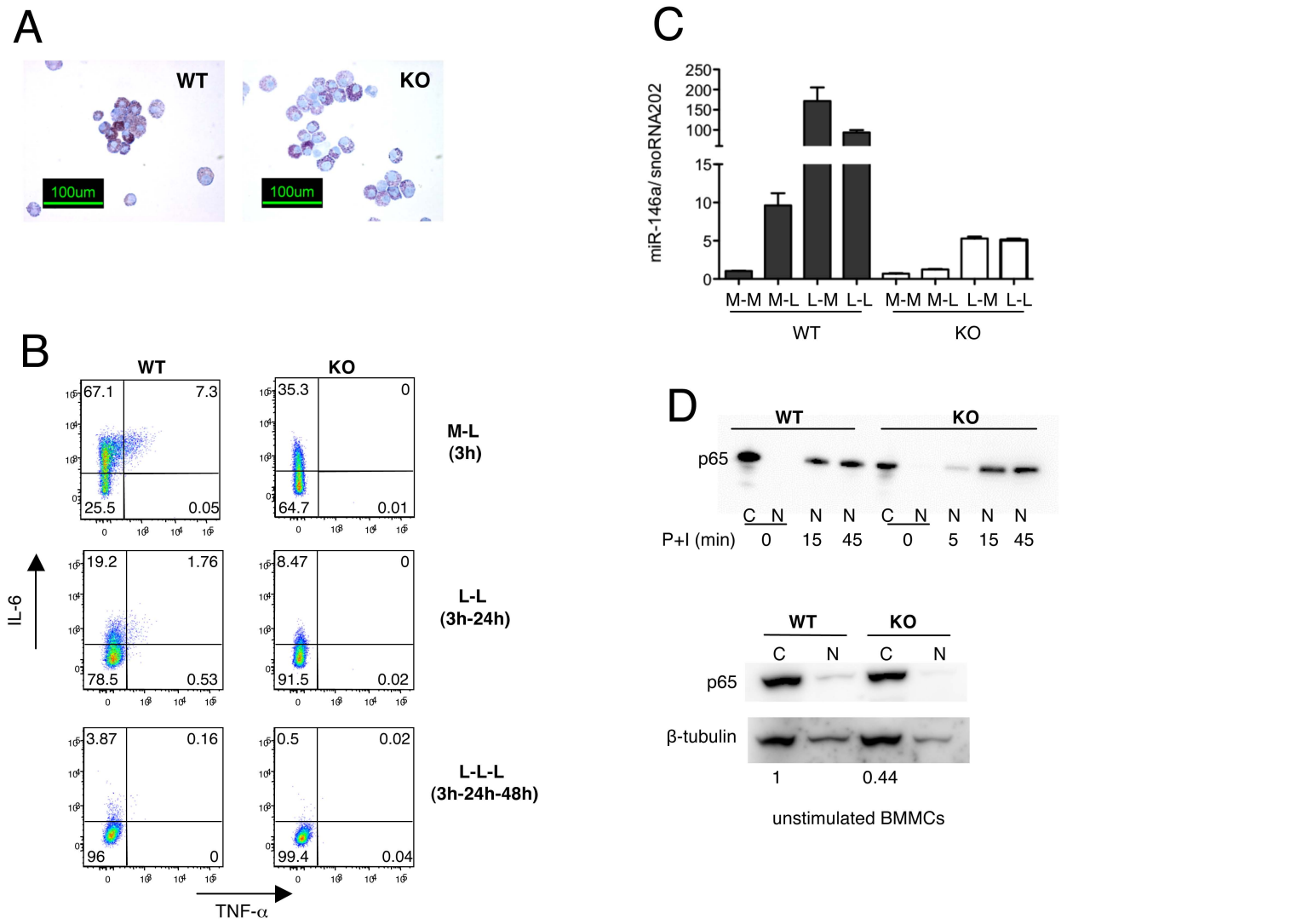
Supplementary Figure 2. MiR-146a is similarly expressed in TH1/ TH2/ TH17 human T cell subsets and does not influence Fas-mediated cell death or CD95 expression. A) MiRNA expression in human T cell subsets. The heat map diagram shows the result of a two-way hierarchical clustering of miRNAs and samples. The color scale shown at the bottom illustrates the relative expression level of a miRNA across all samples: red color represents an expression level above mean, blue color represents expression lower than the mean. **B)** Primary human T cells were transduced with the indicated lentiviral particles and miR-146a expression was assessed by qRT-PCR. **C)** Primary human T cells of different

subsets (mostly TH1 and TH2) were transduced with the indicated lentiviral particles and were either left untreated of were treated over-night with 20ng/mL of an anti-Fas antibody (BioLegend, clone EOS9.1). Shown is the mean (± s.e.m) of 10 independent experiments. **D)** Primary human T cells were transduced with the indicated vectors, and CD95 expression was assessed by surface staining at different days after transduction. Shown is one representative experiment out of three. **E)** Primary human T cells transduced with either a miR-146a- or control-expressing vector were stimulated with PMA and ionomycin for 5h prior intracellular cytokine staining to assess expression of IL-2 and IFN-γ. The staining is representative of tens of experiments.

Supplementary Figure 3. MiR-146a alters T cell expansion in both mouse and human primary T cells and targets TRAF6 expression. A) CD45.1 OTII naïve T cells were FACS-purified and transfected by Amaxa with either a miR-146a mimic oligo or a siGLO negative control, after which cells were injected i.v. into CD45.2 recipient mice. 24h after transfer mice were challenged sub-cutaneously with OVA and the draining lymph node and the spleen were collected and analyzed for CD45.1 expression 3-4 days after challenge. **B)** Naïve T cells transfected as in A) were analyzed for efficiency of transfection (siGLO fluorescence, left) and miR-146a expression (right) 24h after transfection. **C)** Human naïve T cells were FACS-purified, labeled with CFSE and transfected by Amaxa with either a miR-146a mimic oligo or a negative control, after which they were stimulated with irradiated allogeneic-PBMCs. After about 10 days, the percentage of activated, CFSEneg cells was determined by FACS (representative of two independent experiments). **D)** (Top) Schematic rapresentation of the vector used. (Middle panel) Jurkat cells were transduced with a lentiviral vector to express miR-146a (or control vectors expressing either a non-targeting (NT) or irrelevant (shLuc) hairpin), they were selected for several days with puromycin and then further transfected by electroporation with a reporter vector containing the TRAF6 3'UTR

cloned downstream the luciferase reporter gene. 24h after transection the levels of luciferase were assessed by reporter assay. Renilla was used to normalize luciferase expression. (Bottom panel) Primary murine CD4 lymphocytes were differentiated for 5 days in Th2 conditions, after which they were transfected with Amaxa with the reporter pGL3-TRAF6 reporter vector shown on top and with a mimic miR-146a oligo. miR-21 or a mock (no double-stranded RNA) transfection were used as negative controls.

Supplementary Figure 4. Working model on the role of miR-146a and NF-kB p50 in regulating mast cell survival and T cell activation. A) In unmanipulated, acutely stimulated mast cells, NF-kB enters the nucleus where it activates the expression of specific genes, leading to a physiological response. **B)** In the absence of p50, the upregulation of pro-survival genes (A1 and Bcl-2) together with the impaired expression of the negative regulator miR-146a leads to increased survival. **C)** Forced expression of the negative regulator miR-146a leads to reduced NF-kB activity with increased cell death, slight reduction of Bcl-2 expression levels, and partial compensation for the lack of p50. **D)** To become fully activated, a naïve T cells requires a number of accumulating signals that allow the cell to reach a certain 'activation threshold' prior to become fully committed to proliferation and differentiation to effector and memory. We propose that a network comprising miR-146a and p50 is part of these accumulating signals that a naïve T cell requires for appropriate stimulation.



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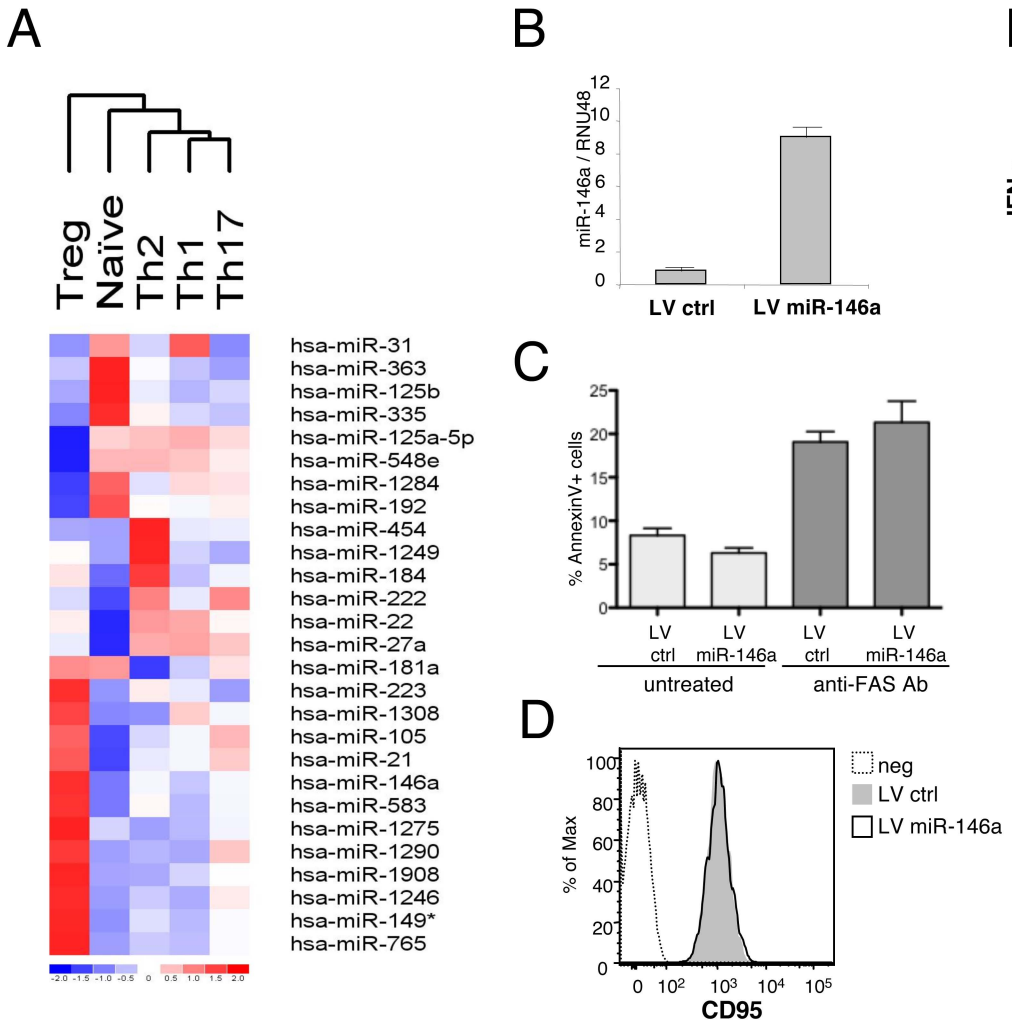

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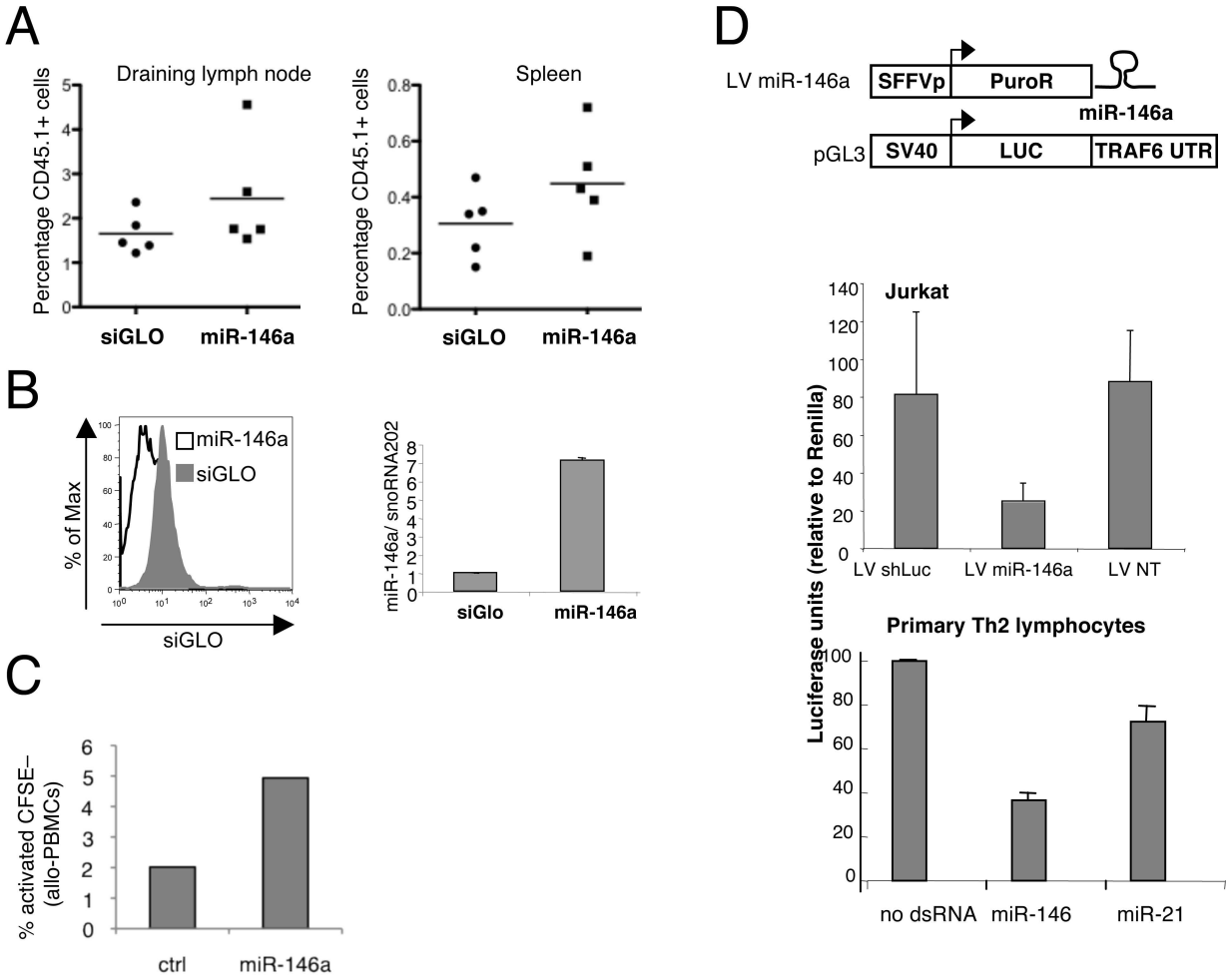

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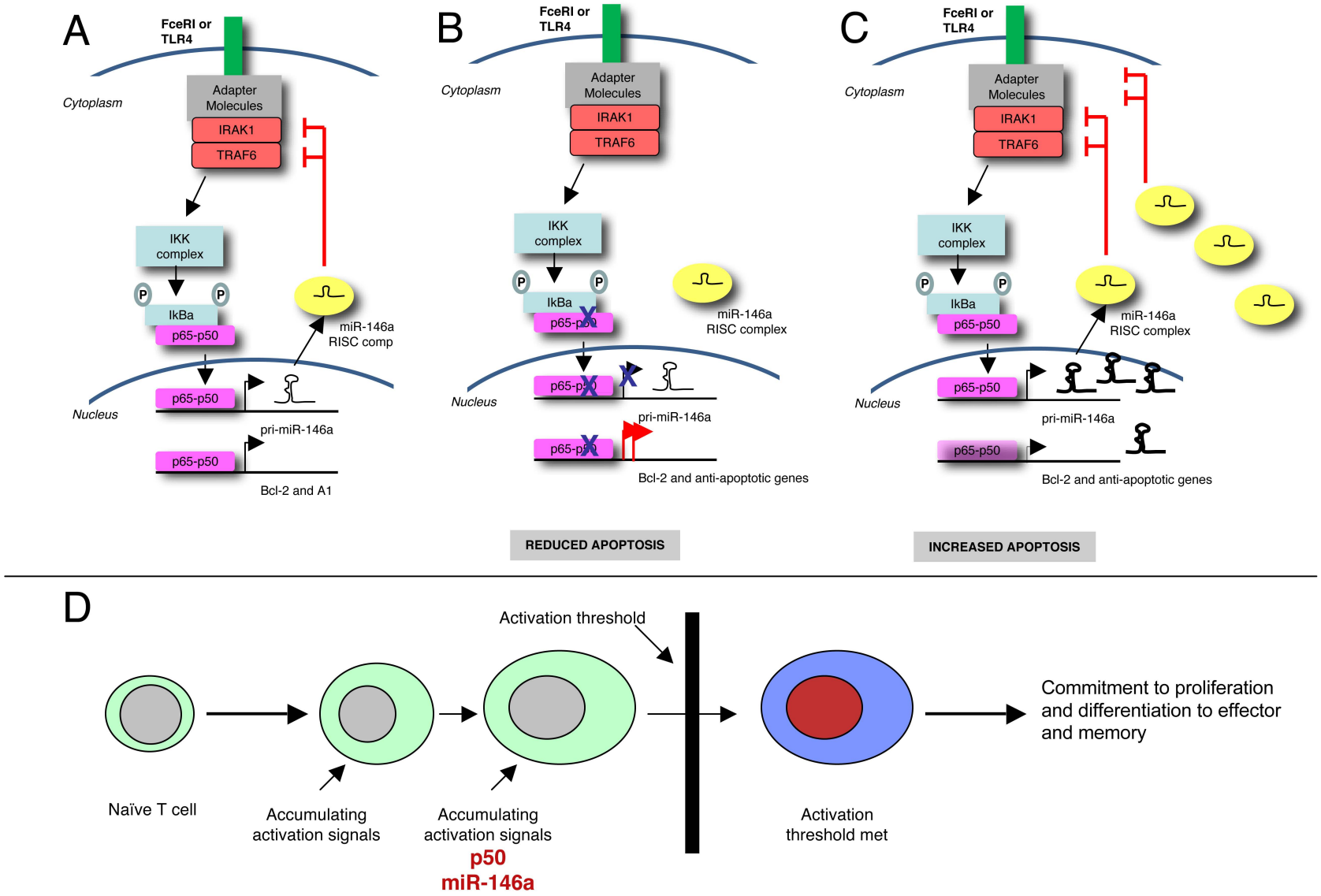

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Supplementary Figure 4